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Activation of histamine H_3 receptor decreased cytoplasmic Ca^{2+} imaging during electrical stimulation in the skeletal myotubes

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ABSTRACT

Histamine is a neurotransmitter and chemical mediator in multiple physiological processes. Histamine H_3 receptor is expressed in the nervous system, heart, and gastrointestinal tract; however, little is known about H_3 receptor in skeletal muscle. The aim of this study was to investigate the role of H_3 receptor in skeletal myotubes. The expression of H_3 receptor and myosin heavy chain (MHC), a late myogenesis marker, was assessed by real-time PCR and immunostaining in C2C12 skeletal myogenesis and adult mid-urethral skeletal muscle tissues. H_3 receptor mRNA showed a significant increase upon differentiation of C2C12 into myotubes: 1-, 26-, 91-, and 182-fold at days 0, 2, 4, and 6, respectively. H_3 receptor immunostaining in differentiated C2C12 cells and adult skeletal muscles was positive and correlated with that of MHC. The functional role of H_3receptor in differentiated myotubes was assessed using an H_3 receptor agonist, (R)-a-methylhistamine ((R)-α-MeHA). Ca^{2+} imaging, stimulated by electric pacing, was decreased by 55% after the treatment of mature C2C12 myotubes with 1 μM (R)-α-MeHA for 10 min and 20 min, while treatment with 100 nm (R)-α-MeHA for 5 min caused 45% inhibition. These results suggested that H_3 receptor may participate in the maintenance of the relaxed state and prevention of over-contraction in mature differentiated myotubes. The elucidation of the role of H_3R in skeletal myogenesis and adult skeletal muscle may open a new direction in the treatment of skeletal muscle disorders, such as muscle weakness, atrophy, and myotonia in motion systems or peri-urethral skeletal muscle tissues.

1. Introduction

Histamine is a well-known biogenic cationic amine that is synthesized, stored, and released by professional histamine-synthesizing cells, such as mast cells, basophils, and enterochromaffin cells. These cells express histidine decarboxylase, which effectively converts L-histidine to histamine (Ichikawa et al., 2010). Histamine plays an important role as an inflammatory mediator and neurotransmitter and exerts its effects by binding to G protein-coupled histamine receptors H_1–H_4 receptor with different affinities. H_1 receptor (pK_i for histamine 4.2) and H_2 receptor (pK_i for histamine 4.3) have long been established as histamine receptors that are expressed in various tissues (Walter and Stark, 2012), but in the last decades, H_3 receptor and H_4 receptor, which have a higher affinity for histamine (pK_i 8.0 and 8.2, respectively), have been characterized mostly in non-professional histamine-producing cells, such as dendritic cells and lymphocytes (Szeberenyi et al., 2001 and Kubo and Nakano, 1999). The data obtained using HR agonists and/or antagonists have suggested that H_4 receptor is mainly involved in immunity and is expressed in immune cells, while H_3 receptor has been reported to be

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expressed in the brain and presynaptic membranes of myoneural junctions (Zampeli and Tiligada, 2009 and Leurs et al., 2005). H_3 receptor activation with a selective agonist in the cardiac sympathetic nerve endings reduced the rise in intracellular Ca^{2+} concentration in response to membrane depolarization, while a selective H_3 receptor antagonist inhibited this effect (Silver et al., 2002). Some studies have also found functional H_3 receptor expressed in smooth muscle cells, such as bronchial smooth muscle cells (Cardell and Edvinsson, 1994) and bladder detrusor cells (Neuhaus et al., 2006). However, there has been no information on H_3 receptor expression in skeletal muscle cells, myoblasts, or myotubes, and, consequently, on the role of H_3 receptor in skeletal myogenesis.

The presence and functional role of H_3 receptor in smooth muscle cells led us to hypothesize that H_3 receptor may also be expressed in skeletal muscles, where it may play a role in cytoplasm and calcium regulation of the myotubes.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 myoblasts were obtained from the Turku Center for Biotechnology, University of Turku, Finland (Pallari et al., 2011), and were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza/BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Boston, MA, USA), antibiotics, and L-glutamine, at 37 °C in a humidified 5% CO_2 atmosphere. The differentiation medium had the same composition, except that FBS was reduced to 1%. The cells were passaged at 80% confluence using trypsinization with 0.5% trypsin in 0.5 mM EDTA (Gibco BRL, Life Technologies, Gaithersburg, MD, USA). Three parallel samples were cultured concurrently.

2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To investigate the expression of H_3 receptor during C2C12 myogenesis, 5×10^4 cells/well were seeded in 12-well plates (CellStar, Greiner Bio-One, Frickenhausen, Germany) in 10% FCS-containing DMEM for 2 days. After reaching 80% confluence, cell monolayers were switched to the differentiation medium to induce myogenesis. Total RNA was isolated from C2C12 cells at days 0, 2, 4, and 6 using the RNeasy Mini Kit (QIAGEN, Düsseldorf, Germany) according to the manufacturer's instructions, and 1 μg of total RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA). qRT-PCR was performed with 100 ng first-strand cDNA as a template, using iQ SYBR Green Supermix (Bio-Rad) and an iCycler iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Primers for the genes encoding mouse myosin heavy chain IIa (MHC-IIa) (forward: 5′-AGCTGCACCTTCTCGTTTGCCA-3′; reverse: 5′-CGGTCAGGGTCGCTCCTGCT-3′; amplicon size 261 bp), H_3 receptor (forward: 5′-TTCGAGCCTCCGCACCCAGAA-3′, reverse: 5′-GGTCCAACGGCCGGTCAGC-3′; amplicon size 118 bp) and porphobilinogen deaminase (PBGD) (forward: 5′-AAAGTGCCGTGGGAACCAGC-3′, reverse: 5′-CAGCCACAGCCAGGACGATG-3′; amplicon size 156 bp) were designed using the NCBI Primer BLAST program. The mRNA copy numbers in the samples were determined in triplicate and were normalized against that of PBGD. The results were analyzed using the comparative CT method and the expression levels of the cells of each individual were normalized to the expression of D0.

2.3. Immunofluorescence

C2C12 cells were seeded at a density of 2×10^4 cells/well on coverslips in 24-well plates (Cell Star, Sigma-Aldrich, St Louis, MO, USA) and allowed to grow in DMEM with 10% FCS for 2 days to reach 80% confluence; then, the cells were switched to differentiation medium to induce myogenesis. The cells from differentiation days 0 and 6 were fixed in 4% paraformaldehyde in 10 mM phosphate-buffered 140 mM saline (PBS, pH 7.4) for 15–20 min, washed three times for 5 min in PBS and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Cells were blocked with 10% normal donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 h and incubated at 4 °C overnight with 1 μg/ml of primary antibodies: polyclonal affinity-purified rabbit anti-human MHC IgG (a gift of Dr. John E. Erikson, University of Turku, Turku, Finland) (Pallari et al., 2011), rabbit anti-human H_3 receptor polyclonal antibodies (LS-A476, MBL International, Woburn, MA, USA), or non-immune rabbit IgG (R&D Systems, Minneapolis, MN, USA) was used as negative control. Cells were washed three times for 5 min in PBS and incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) diluted 1:400 in 0.1% BSA–PBS for 1 h, washed three times for 5 min in PBS each time, and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in distilled water, for 5 min. The coverslips were washed quickly twice in PBS and distilled water for 10 min before mounting with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Labeled slides were photographed using a Leica DM 6000 B/M fluorescence microscope equipped with a motorized Leica XY-stage and connected to a Leica DFC 420 digital camera (Leica Microsystems, Wetzlar, Germany) and analyzed using the Leica Application Suite Advanced Fluorescence 2.5.0.6735 software.

2.4. Immunohistochemistry

The study protocol was approved by the institutional Medical Ethics Committee and was in accord with the 1983 Declaration of Helsinki. Informed consent was obtained from all volunteers.

The mid-urethral striated muscle samples were obtained from three healthy male volunteers by biopsy. The samples were fixed in 40% formaldehyde solution for 24 h and embedded in paraffin. Paraffin blocks were sliced into 3–5-μm sections, which were placed on the slides and incubated at 37 °C overnight. Samples were deparaffinized by using xylene and antigens were retrieved by heating samples in a microwave in 10 mM citrate buffer, and were then incubated in 1% hydrogen peroxide to quench endogenous peroxidase activity. The samples were stained using a VECTASTAIN Elite ABC Kit (Rabbit IgG) (PK-6101, Vector Laboratories, Burlingame, CA, USA). The slides were blocked in 10% normal donkey serum for 1 h and incubated at 4 °C overnight with 1 μg/ml of primary antibodies, viz., polyclonal affinity-purified rabbit anti-human MHC IgG, rabbit anti-human H_3 receptor polyclonal antibodies, or non-immune rabbit IgG used as negative control. The slides were washed three times for 5 min in PBS and were then incubated with the secondary donkey anti-rabbit IgG diluted 1:400 in 0.1% BSA–PBS for 1 h. After washing three times for 5 min in PBS, the slides were incubated with the ABC complex for 1 h, washed three times for 5 min in PBS, and were then incubated in the peroxidase substrate solution until the desired staining intensity appeared (maximum time: 10 min). The slides were then washed in distilled water, counterstained in Mayer's hematoxylin, washed in running water, dehydrated in graded ethanol, and cleared in xylene before being mounted in Vectashield.

2.5. Cytoplasmic Ca^{2+} imaging

C2C12 cells were differentiated for 7 days into myotubes on 18-mm coverslips. The cells were then loaded with 4 μmol/L Fluo-4 AM Ca^{2+} indicator dye (F-14201, Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature in Elliot medium, containing (mM): 137 NaCl, 5 KCl, 0.44 KH_2PO_4, 20 HEPES, 4.2 $NaHCO_3$, 5 D-glucose, 2 $CaCl_2$, 1.2 $MgCl_2$, and 1 Na-pyruvate (pH adjusted to 7.4 with NaOH). Then, a 30-min de-esterification was performed at 37 °C and the coverslips were transferred to an RC-49MFS recording chamber equipped with electrodes for field stimulation (Warner Instruments Inc., Hamden, CT, USA) and maintained at 37 °C during imaging.

H_3 receptor agonist R-(a)-methylhistamine ((R)-α-MeHA), at different concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM) in Elliot medium, and a blank control (Elliot medium) were added into parallel coverslip chambers for 5, 10, 20 min, and 30 min before electrical pacing. Ca^{2+} imaging in the cytoplasm was recorded during stimulation. The cells were electrically paced at a frequency of 0.5 Hz with 10-ms bipolar pulses (200–360 mA) using a SIU-102 Stimulus Isolation Unit (Warner Instruments, Hamden, CT, USA) connected to a Master-8 stimulator (A.M.P.I., Jerusalem, Israel). Ca^{2+} imaging was observed under an inverted IX70 microscope (Olympus Corporation, Hamburg, Germany) equipped with transparent housing, a heater, and a temperature control unit (Solent Scientific, Ltd, Segensworth, UK) using a UPlanSAPo 20× air objective (Olympus Corporation, Hamburg, Germany). Polychrome IV (TILL Photonics, Graefelfing, Germany) was used to illuminate the sample at a wavelength of 488 nm; emitted light was filtered through a Chroma filter set (EM HQ535/50 m, BS Q505LP, Chroma Technology Corporation, Bellows Falls, VT, USA). Images were recorded with a Hamamatsu ORCA-Flash 4.0 sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan) at 50 frames/s. Data acquisition was controlled and the recordings were analyzed using HC Image software (Hamamatsu Photonics K.K., Hamamatsu, Japan). The cells for analysis were manually selected in the regions of interest and the background was subtracted before quantifying the fluorescence relative to baseline fluorescence (F/F_0).

2.6. Statistical analysis

All values are given as mean ± standard error of the mean (S.E.M.). Statistical differences were evaluated using the Mann–Whitney test (Matlab, MathWorks Inc., Natick, MA, USA).

3. Results

3.1. C2C12 cell morphology during myogenesis

Before differentiation, C2C12 cells presented as mononuclear myoblasts around 10-μm in diameter, round or spindle-like; they were well spread and showed many contacts with the substrate (Fig. 1A). When the cells were induced to differentiate, they started proliferating and migrating and then arranged themselves along a single direction to fuse into myotubes. After 6 days of differentiation, the cells fused into long, multinucleated myotubes of over 150-μm in diameter (Fig. 1B).

3.2. H_3 receptor expression during myogenesis and in human mid-urethral striated muscles

The expression of H_3 receptor and the late myogenesis marker MHC during C2C12 differentiation was assessed using qRT-PCR. MHC increased over the course of differentiation by 7168-, 89487-

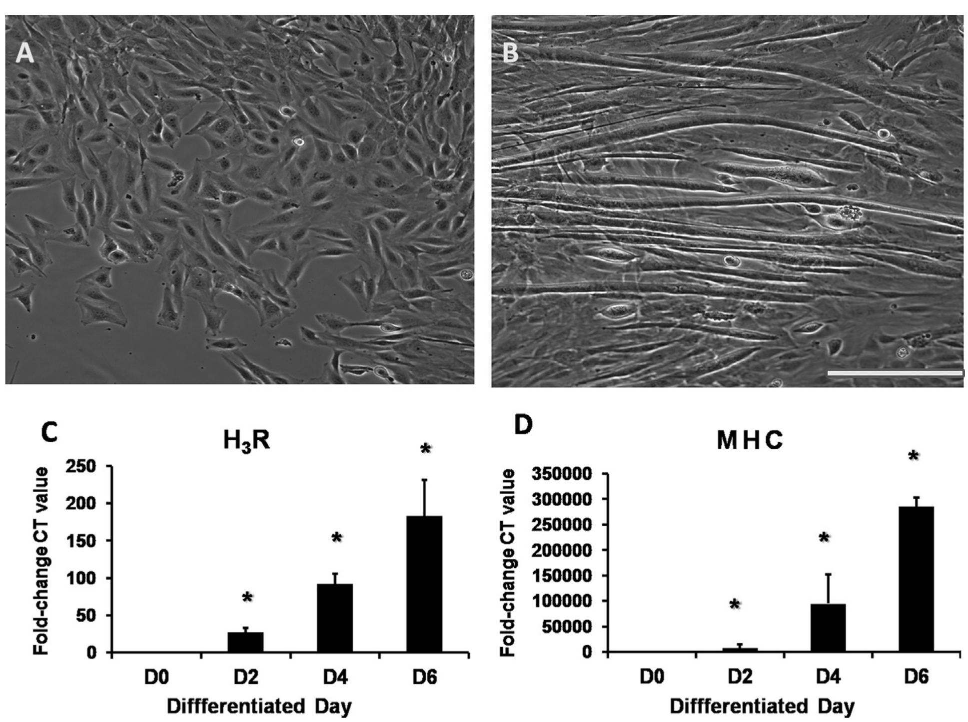

Fig. 1. C2C12 cell differentiation and H_3 receptor expression. C2C12 cells grown in 10% fetal calf serum (FCS)-containing DMEM were switched to 1% FCS DMEM and differentiated for 6 days. The expression of H_3 receptor and MHC (a differentiation marker) was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (A) C2C12 myoblasts at differentiation day 0; (B) C2C12 myotubes at differentiation day 6; (C) H_3 receptor expression during myogenesis at days 2, 4, and 6; (D) MHC expression during myogenesis at days 2, 4, and 6. The scale bar represents 50 μm. Data are expressed as the mean ± standard deviation (S.E.M.), and the number of independent experiments is three; $P < 0.01$ compared to day 0 (D0). Typically this is indicated with a *. The qRT-PCR results of the cells of each individual were normalized to the expression at D0, and the expression of D0 is set to 1.

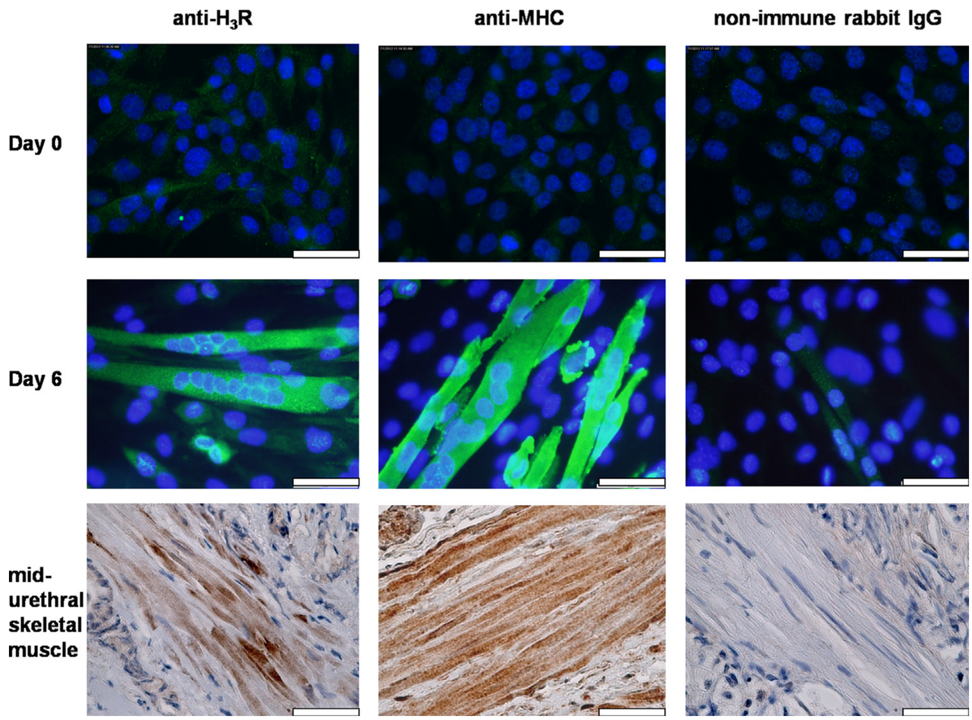

Fig. 2. Immunostaining of H_3 receptor and MHC in differentiated C2C12 cells and adult mid-urethral skeletal muscle tissues. C2C12 cells were grown in 10% FCS-containing DMEM; the medium was then switched to 1% FCS DMEM and differentiated for 6 days. C2C12 cells and mid-urethral skeletal muscle tissues were fixed in 40% formaldehyde solution and stained with the anti-H_3 receptor antibody, anti-MHC antibody, and non-immune rabbit IgG. Upper panel: C2C12 cells, differentiation day 0 (Day 0); middle panel: C2C12, differentiation day 6 (Day 6); lower panel: mid-urethral skeletal muscle tissues. C2C12 cells were counterstained with DAPI and mid-urethral skeletal muscle tissues were counterstained with Mayer's hematoxylin, to reveal cell nuclei (blue). The scale bar represents 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and 279897-fold at days 2, 4, and 6, respectively, compared to day 0 (Fig. 1D). The expression of H_3 receptor, which was initially low in C2C12 myoblasts, also strongly increased with differentiation when compared to day 0; by 26-, 91-, and 182-fold at days 2, 4, and 6, respectively (Fig. 1C).

The immunofluorescence staining of the MHC protein was negative before differentiation (Fig. 2), but was strongly positive at day 6 (Fig. 2). Similarly, immunostaining for the H_3 receptor protein was negative at day 0 (Fig. 2), but was strongly positive at day 6 (Fig. 2).

Human mid-urethral striated muscles showed positive immunostaining for both MHC and H_3 receptor proteins in the filiform-like muscle cells (Fig. 2).

3.3. *The H_3 receptor agonist (R)-α-MeHA decreased cytoplasmic Ca^{2+} imaging evoked by electrical stimulation*

(R)-α-MeHA of different concentrations was added to stimulate 7 day-differentiated myotubes for 5, 10, and 20 min prior to electrical pacing, and the Ca^{2+} imaging in the cytoplasm was assessed during stimulation (Fig. 3). Incubation with 1 μM (R)-α-MeHA for 10 or 20 min significantly (55%) decreased the increase of cytoplasmic Ca^{2+} imaging upon electrical pacing. Shorter (5 min) incubation with 100 nM (R)-α-MeHA decreased Ca^{2+} imaging by 45%; longer treatments further increased the decrease-effect when (R)-α-MeHA was over 100 nM. However, (R)-α-MeHA activity in C2C12 cells displayed bell-shaped dynamics: the concentration increase over 1 μm significantly reduced the decrease-effect of intracellular Ca^{2+} imaging upon electrical stimulation (Fig. 3). The result of (R)-α-MeHA treatment at 30 min is not shown, because obvious decay of the Ca^{2+}-labeled Fluo-4 was detected at this time.

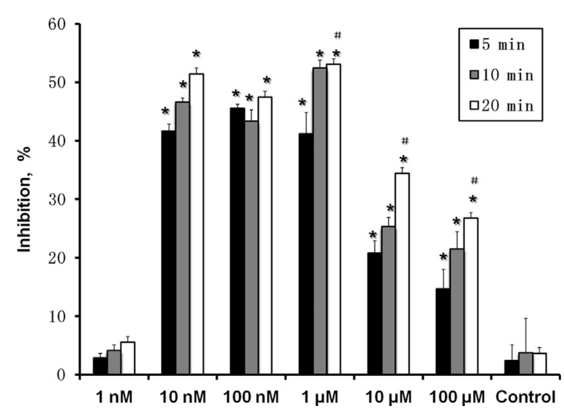

Fig. 3. H_3 receptor agonist decreased cytoplasmic Ca^{2+} imaging during electrical stimulation in the skeletal myotubes. C2C12 myotubes, differentiated for 7 days, were loaded with Fluo-4 AM Ca^{2+} indicator dye and were then incubated with the H_3 receptor agonist (R)-α-MeHA at different concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM) and blank control (Elliot medium) for 5, 10, and 20 min before electric pacing. Ca^{2+} imaging was performed as described in Section 2. The data are expressed as the mean ± standard deviation (S.E.M.), the number of independent experiments is 3, and $P < 0.01$ compared to control is indicated; typically this is indicated with a *.It is $P < 0.05$ compared to 5 min results; typically this is indicated with a ♯.

4. Discussion

To our knowledge, this is the first investigation of the role of H_3 receptor in skeletal muscle cells. When we first observed H_3 receptor immunostaining in adult skeletal muscle, we hypothesized that H_3 receptor may play a role in skeletal myogenesis or mature skeletal muscle cells. To test this proposal, we assessed H_3 receptor expression kinetics during differentiation of mouse C2C12 myoblast cells. H_3 receptor expression was almost absent in myoblasts, but increased markedly with myogenesis, suggesting that H_3 receptor mostly functions in differentiated myoblasts that were fused into myotubes. H_3 receptor functional analysis, using an H_3 receptor agonist (R)-α-MeHA, revealed that in fully differentiated 7-day myotubes, H_3 receptor activation decreased Ca^{2+} imaging evoked by electrical stimulation. The lowest (R)-α-MeHA concentration used (1 nM) was below the agonist affinity for H_3 receptor ($pK_i = 8.4$) in the brain (Chen et al., 2003 and Li et al., 2014) and had no effect on the cytoplasmic Ca^{2+} imaging under electrical stimulation in our study. On the other hand, the highest (R)-α-MeHA concentration used (100 μM) was also less active, probably because of combinatorial effects, due to nonspecific activation of other receptors (H_1 receptor or H_2 receptor) on the cell membrane (Chen et al., 2015), which counteracted the response (Leurs et al., 1998), or probably as a self-protective mechanism. The highest inhibitory effect of 1 μM (R)-α-MeHA, exerted after 10- min or 20 min treatment, was in agreement with a previous pharmacological study on histamine receptor ligands, where (R)-α-MeHA also showed the highest efficacy at 1 μM (Kottke et al., 2011).

The histamine receptors, including H_3 receptor, are a class of G-protein-coupled receptors. H_3 receptor is coupled to the $G_{i/o}$ proteins, which have prominent effects on Ca^{2+} influx. H_3 receptor-mediated activation of $G_{i/o}$ proteins may cause the activation of phospholipase A_2 (PLA_2), which induces the release of arachidonic acid and the inhibition of the Na^+/H^+ exchanger, leading to a decrease in intracellular Ca^{2+} levels involving the impaired entrance of Ca^{2+} through voltage-gated ion channels (Leurs et al., 2005). In cardiac sympathetic nerves, H_3 receptor-mediated inhibition of norepinephrine exocytosis was caused by H_3 receptor-G_i/G_o coupling, and inhibition of adenylyl cyclase activity and cAMP formation, leading to diminished protein kinase A (PKA) activity, and decreased Ca^{2+} influx through voltage-operated Ca^{2+} channels (Leurs et al., 2005). In our experiments, H_3 receptor activation in the differentiated C2C12 myotubes decreased intracellular Ca^{2+} imaging in response to electrical pacing, which is similar to the effect observed in cardiac sympathetic nerve endings.

In mature skeletal muscle cells, the major source of Ca^{2+} is the intracellular Ca^{2+} store in the sarcoplasmic reticulum (SR), which accumulates Ca^{2+} from the cytoplasm in an ATP-dependent manner by the action of Ca^{2+}-ATPase (Endo, 2009). Most of this calcium moves back and forth across the SR membrane in cycles of contraction and relaxation. The channel responsible for release from the SR is the ryanodine receptor (RyR). The RyR that mediates the efflux of Ca^{2+} from the SR has a central role in excitation–contraction coupling between sarcolemmal depolarization and SR Ca^{2+} release (Stokes and Wagenknecht, 2000).

In early studies of isolated frog skeletal muscles, catecholamines induced sarcolemmal-depolarized contraction through elevation of cAMP, which also stimulates RyR on the SR, and increased Ca^{2+} flux out from the SR into the cytoplasm (Gonzalez-Serratos et al., 1981). Regulation of Ca^{2+} concentration in the cytoplasm is important in coupling excitation and contraction in skeletal muscle (Emrick et al., 2010). Evidence has suggested that the level of cyclic AMP may be involved in the increase of Ca^{2+} in the cytoplasm (Ong and Steiner, 1977 and Lanner et al., 2010) and is associated with contraction of both cardiac and skeletal muscles (Gonzalez-Serratos et al., 1981). In cultured skeletal myotubes, the activated calcitonin gene-related peptide (CGRP) receptor can positively couple to Gs proteins and activation of adenylate cyclase, increasing levels of cAMP generation and subsequent protein kinase A (PKA) activation to increase contraction force. During this process, CGRP increases voltage-gated SR Ca^{2+} release within hours, resulting from a quantitatively similar increase in releasable SR Ca^{2+} content (Avila et al., 2007). When chicken and rat skeletal muscle cells were cultured for 7 days and were then subjected to electrical stimulation for additional 2 days, the ability of these cells to synthesize cAMP was increased by almost two-fold (Young et al., 2000). In our study, electrical stimulation of differentiated C2C12 myotubes may have caused upregulation of cAMP biosynthesis; in this case, H_3 receptor activation may inhibit cAMP elevation and prevent over-contraction. Other studies found histidine decarboxylase activity was very low in normal non exercised muscle (Endo et al., 1998) and mice treated with a H_3 receptor antagonist display no significant effects on prolonged walking endurance (Niijima-Yaoita et al., 2012), suggesting that histamine and H_3 receptor signaling have a limited role on normal muscle physiology. It is the possibility that H_3 receptor is activated only in exercised and/or excited muscle. In addition, we also tried ciproxifan (histamine H_3-receptor antagonist, $pK_i = 9.1–9.4$) 1 μM instead of (R)-α-MeHA to do the experiment, and found there was no cytoplamic Ca^{2+} imaging arise compare to the control. So H_3 receptor blockage might not enhance cell contraction.

In conclusion, we found that the histamine H_3 receptor agonist (R)-α-MeHA can attenuate cytoplasmic Ca^{2+} imaging during electrical stimulation in differentiated C2C12 myotubes, which may facilitate relaxation of cells and prevent over-contraction in electrically stimulated C2C12 myotubes. Attenuation of the cytoplasmic Ca^{2+} imaging may occur via activation of H_3 receptor to induce inhibition of cAMP formation, or activation of PLA_2, release of arachidonic acid, and the subsequent decrease in intracellular Ca^{2+} levels. These effects are most prominent early during myotube maturation. Whether these effects also occur in muscle regeneration remains to be elucidated.

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